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Mitochondrial DNA mutations in exhaled breath condensate of patients with lung cancer

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Breath test

Summary
Introduction: Lung cancer is a leading cause of cancer mortality worldwide. Non-invasively collected biofluids such as exhaled breath condensate (EBC) present a potential sampling medium to detect and study pathological changes implicated in tumourigenesis. Mitochondrial DNA changes have been implicated in the carcinogenesis process. Consequently, the detection of mitochondrial changes in EBC could expand our understanding of lung carcinogenesis as well as identifying specific markers for future studies.
Methods: EBC and saliva was collected from newly diagnosed subjects with lung cancer and control subjects in a cross-sectional study. The EBC and saliva was analysed for mitochondrial DNA D-loop changes using a PCR sequencing approach. The sequences obtained were compared to paired salivary DNA and the revised Cambridge Reference Sequence (rCRS) to identify somatic mutations, and quantitative and qualitative differences in mutations were analysed between groups.
Results: A total of 25 subjects (9 NSCLC patients, 10 smokers/ex-smokers and 6 non-smokers) were recruited. A significantly elevated D-loop mutation rate in the lung cancer group compared to the control groups was present (7 vs 3.5 for smokers/ex-smokers, and 7 vs. 4 for non-smokers, $p = 0.034$). The recognised mutation T16217C showed specificity for lung cancer.
Conclusions: Mitochondrial DNA mutations are more common in the EBC of patients with lung cancer. This suggests that these processes are associated with the carcinogenesis of lung cancer and may be a marker of the disease.

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Introduction

Lung cancer remains the leading cause of cancer death globally, accounting for 13% of all deaths.[1] The low 5-year survival rate of 15% can be attributed to late diagnosis in the vast majority of cases,[2] for which there are suboptimal therapies. Exhaled breath condensate (EBC) is a non-invasive method of sampling the respiratory epithelial lining fluid.[3] Genetic[4–7] and epigenetic[8] changes in EBC of patients with lung cancer have demonstrated mixed results, but these changes may be informative markers of disease[9] and recently tissue studies have highlighted the potential role of mitochondrial DNA changes in carcinogenesis and as biomarkers of lung cancer.

Mitochondrial DNA (mtDNA) is a 16,569 base pair (bp) circular double-stranded molecule coding for 37 genes including 13 polypeptides involved in respiration and oxidative phosphorylation.[10] mtDNA is more susceptible to oxidative damage than nuclear DNA due to its proximity to the electron transport chain, inefficient repair mechanisms and lack of protective histones.[11–13] Somatic mtDNA mutations have been hypothesised to play a part in cancer through pathways such as mitochondrial respiratory chain dysfunction which increases reactive oxygen species (ROS) production,[14] interference with cellular apoptosis[15,16] and translocation causing activation of nuclear oncogenes, although recent studies have identified anti-tumorigenic mtDNA mutations as well.[17]

mtDNA mutations accumulate in the D-loop region, a 1,124bp non-coding region containing the major control elements for mtDNA expression, replication and transcription.[18] Studies of lung tissue, BAL and sputum have demonstrated that D-loop mutations are prevalent in lung tumours[19–27] and correlate weakly with clinical characteristics such as tumour grade and lymph node metastasis.[27]

EBC may be a non-invasive method of detecting mitochondrial markers of lung cancer. Due to its high copy number and high mutation rate, mutations in the D-loop region of mtDNA are ideal to study in low-cellularity samples like EBC, and may play a role in contributing to the genetic instability involved in carcinogenesis.[28] Specifically, EBC mtDNA may reflect changes observed in studies of lung tissue and it was hypothesised that a higher somatic mutation rate in patients with lung cancer would be observed when compared with healthy control subjects.

Materials and methods

Subject recruitment

This cross-sectional observational study was granted ethics approval (SESAHS HREC04/179). Subjects were recruited from the Prince of Wales Hospital (POWH), and gave written, informed consent. The study population had a confirmed histological diagnosis of non small cell lung carcinoma (NSCLC) by bronchoscopic biopsy or transthoracic cytological aspiration and were enrolled prior to treatment. Control subjects were recruited from the inpatient, outpatient and community populations. Exclusion criteria: history of chronic respiratory diseases, $FEV_1/FVC<70\%$, known history of lung cancer and recent or current respiratory illness (<2 weeks). Demographic and medical details were obtained via a questionnaire. Ex-smokers were those who had ceased smoking for >1 year.

EBC and saliva collection

Subjects refrained from smoking, eating or drinking 2 h prior to EBC collection. After rinsing their mouths with water, subjects provided a saliva sample (average volume 0.5 mL) which was stored at −80 °C.

EBC was collected using a custom-made glass condenser as previously described[29,30]

Subjects breathed through a unidirectional valve, at a normal frequency and tidal volume, for 20 min. EBC was collected on ice at 4 °C, aliquoted and deaerated with argon degassing at 0.4L/min for 1 min, and immediately stored at −80 °C. Unpublished studies demonstrated that this method does not allow salivary DNA contamination or cross-over DNA contamination.

Nucleic acid isolation

EBC (minimum volume 400 μL; average 1000 μL) was concentrated to 200 μL using a SpeedVac SC110A (Thermo Scientific, Victoria, Australia). DNA was extracted from the concentrated EBC using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with modifications to increase DNA yield and concentration; DNA was eluted in 55 μL of pre-heated buffer AE and the eluate was passed through the column again in a second elution step. DNA was extracted from saliva using the same kit using a 100 μL elution volume. The resulting EBC-DNA and saliva-DNA either immediately underwent PCR amplification or was stored at −20 °C.

Polymerase chain reaction amplification

A 1450 base-pair (bp) segment of the mitochondrial genome containing the D-loop region was amplified using the forward primer 5′-TACTCAAATGGGCCTGTCCT-3′ and reverse primer 5′-AGGGTGAACTCACTGGAACG-3′. These primers were custom-designed, based on the revised Cambridge Reference Sequence (rCRS, accession number: NC_012920), using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) and submitting to BLAST search (Basic Local Alignment Search Tool, www.ncbi.nlm.nih.gov/BLAST/). Primers were purchased from Invitrogen (Carlsbad, CA, USA).

PCR was performed in 50 μL reactions with 15 μL of EBC-DNA, 2.5U of Fast Start High Fidelity Taq (Roche Applied Science, Mannheim, Germany) in supplied PCR buffer, 0.4 mM of each primer, 1.5 mM Mg^{2+}, 200 μM of deoxyribonucleoside triphosphate (dNTPs) and 5% DMSO. Thermal cycling was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Victoria, Australia) with an initial denaturing step at 95 °C for 2 min, followed by 42 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min 15 s, and a final extension at 72 °C for 7 min. Salivary DNA PCR was performed on 30 ng of sample in a final volume of 25 μL using the same reaction